Nontranscriptional Function of FOXO1/DAF-16 Contributes to Translesion DNA Synthesis

Hiroaki Daitoku,^{a,b} Yuta Kaneko,^b Kenji Yoshimochi,^b Kaori Matsumoto,^b Sho Araoi,^b Jun-ichi Sakamaki,^{b*} Yuta Takahashi,^a Akiyoshi Fukamizu^{a,b}

Life Science Center, Tsukuba Advanced Research Alliance,^a and Graduate School of Life and Environmental Sciences,^b University of Tsukuba, Tennoudai, Tsukuba, Ibaraki, Japan

Forkhead box O (FOXO; DAF-16 in nematodes) transcription factors activate a program of genes that control stress resistance, metabolism, and life span. Given the adverse impact of the stochastic DNA damage on organismal development and aging, we examined the role of FOXO/DAF-16 in UV-induced DNA damage response. Knockdown of FOXO1 but not of FOXO3a increases sensitivity to UV irradiation when exposed during S phase, suggesting a contribution of FOXO1 to translesion DNA synthesis (TLS), a replicative bypass of UV-induced DNA lesions. Actually, FOXO1 depletion results in sustained activation of ATR-Chk1 signaling and a reduction of proliferating cell nuclear antigen (PCNA) monoubiquitination following UV irradiation. FOXO1 does not alter the expression of TLS-related genes, but it binds to replication protein A 1 (RPA1), which coats single-stranded DNA and acts as a scaffold for TLS. In *Caenorhabditis elegans*, *daf-16*-null mutants show UV-induced retardation in larval development and are rescued by overexpressing a DAF-16 mutant lacking the transactivation domain but not a mutant whose amino acid substitutions render it unable to interact with RPA1. Thus, our findings demonstrate that FOXO1/DAF-16 is a functional component in TLS independent of its transactivation activity.

The forkhead box O (FOXO) family of transcription factors play crucial roles in diverse biological processes, orchestrating programs of gene expression that regulate cell cycle progression, metabolism, and stress resistance (1). In mammals, the FOXO family consists of four members, FOXO1, FOXO3, FOXO4, and FOXO6, while invertebrates have only one FOXO gene, such as *dFOXO* in *Drosophila* flies and *daf-16* in *Caenorhabditis elegans*. FOXO proteins are tightly regulated by multiple posttranslational modifications, including phosphorylation, acetylation, ubiquitination, and arginine methylation (2, 3). Among them, a major form of regulation is Akt-mediated phosphorylation, which is downstream from the insulin or insulin-like growth factor 1 (IGF-1) signaling pathway and results in the export of FOXO proteins from the nucleus to the cytoplasm, thereby repressing the transcription of FOXO target genes (4). Genetic studies in *C. elegans* have revealed that reduction-of-function mutations of *daf-2*, an ortholog of the mammalian insulin/IGF-1 receptor, extend the life span up to threefold, and this extension is entirely dependent on *daf-16* (5). Accordingly, gene expression regulated by DAF-16 is considered to be a trigger for promoting antiaging and longevity, and to date, a number of approaches have identified various DAF-16 target genes that control detoxification, lipolysis, and autophagy (6, 7). In addition, although accumulating evidence indicates that aging is accompanied by an increase in genomic instability (8, 9), the involvement of FOXO/DAF-16 in the DNA damage response remains largely unknown.

Among the numerous harmful agents, UV irradiation is a ubiquitous environmental stress, which generates two types of DNA lesions, cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidine photoproducts (6-4PP). These UV-induced DNA lesions are usually removed by one of the most versatile DNA repair systems, called nucleotide excision repair (NER), whereas if left unrepaired until S phase, they interfere with the progress of replication forks catalyzed by DNA polymerases (10). In order to prevent the collapse of stalled replication forks,

which in turn leads to DNA double-strand breaks, translesion DNA synthesis (TLS) is performed by the Y family DNA polymerases, which include polymerases eta (Pol η), kappa (Pol κ), and iota (Pol ι) and Rev1 (11). When replication forks block at CPD sites, Pol η has been shown to replace the stalled replicative DNA polymerases by a mechanism that is dependent on monoubiquitination of the clamp protein, proliferating cell nuclear antigen (PCNA), by the E3 ubiquitin ligase RAD18. This modification is activated by single-stranded DNA (ssDNA) coated with the ssDNA-binding protein replication protein A (RPA), whereby monoubiquitinated PCNA has an increased affinity for Pol η , thus helping to recruit Pol η to stalled replication forks and allowing accurate replicative bypass of CPD by incorporating correct bases on the opposite strand (12, 13). Together with PCNA, the clamp loader replication factor C (RFC) complex and RPA have been shown to stimulate the DNA-synthetic activity of Pol η (14–16). On the other hand, defects in Pol η result in a cancer-prone and UV-sensitive inherited syndrome, a variant form of xeroderma

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Address correspondence to Akiyoshi Fukamizu, akif@tara.tsukuba.ac.jp.

* Present address: Jun-ichi Sakamaki, The Beatson Institute for Cancer Research, Bearsden, Glasgow, United Kingdom.

H.D. and Y.K. contributed equally to this work.

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pigmentosum, suggesting that Pol η is essential for preventing UV-induced skin cancers (17, 18).

In this study, we show that FOXO1 is involved in tolerance of UV-induced DNA damage across species. In mammalian cells, knockdown of FOXO1 but not of FOXO3a increases sensitivity to UV specifically when cells are irradiated during S phase. FOXO1 depletion also abrogates the progression of S phase after UV irradiation and thereby inhibits cell proliferation. These results led us to hypothesize that FOXO1 would be implicated in the TLS process. Supporting our hypothesis, knockdown of FOXO1 results in sustained activation of the ataxia telangiectasia and Rad3-related protein (ATR)–Chk1 pathway and reduction of PCNA monoubiquitination. FOXO1 does not alter the expression of TLS-related genes, but it binds to RPA1 through the forkhead domain and this binding enables FOXO1 to target an ssDNA *in vitro*. In addition, we find that FOXO/DAF-16 plays a key role in the tolerance of UV-induced DNA damage in nematodes. Compared to wild-type (WT) controls, *daf-16*-null mutants showed a UV-induced delay in larval development, while UV irradiation had no effect on the adult life span, suggesting that DAF-16 confers UV tolerance only during somatic cell proliferation in *C. elegans*. Furthermore, rescue experiments revealed that the transactivation function of DAF-16 is not necessary for the UV tolerance in larval development. Taken together, our findings demonstrate an evolutionally conserved and transactivation-independent FOXO1/DAF-16 function in the tolerance of UV-induced DNA damage that may contribute, at least in part, to genomic stability by preventing stalled replication forks from degenerating into deleterious DNA structures.

MATERIALS AND METHODS

Cell culture, transfection, and siRNA. HEK293, HEK293T, HeLa, and MCF7 cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Plasmid transfection was performed using GeneJuice transfection reagent (Novagen) according to the manufacturer's protocol. Small interfering RNA (siRNA) duplexes were synthesized by Nippon EGT and transfected using Lipofectamine RNAiMAX (Invitrogen). The siRNA sequences were as follows: human FOXO1, 5'-AGUUCAUUCGUGUGCAGAATT-3' (siFOXO1 no. 1) and 5'-AGAGCUGCAUCCAUGGACATT-3' (siFOXO1 no. 2); siPol η , 5'-GUGGAGCAGCGGCAAAUUCTT-3'; siFOXO3a, 5'-GAGCTCTTGGTGGATCATC-3'; siGADD45 α , 5'-GGAUCCUGCCUUAAGUCAACUUAUU-3'; and luciferase, 5'-UAAGGCUAUGAAGAGAUACTT-3' (siCont no. 1).

The following siRNAs were purchased: siRNA against Rad18 (sc-72142; Santa Cruz) and control siRNA against green fluorescent protein (GFP) (siCont no. 2) (S10C-0300; B-Bridge) (5'-CUACAACAGCCACAACGUC-3').

TUNEL assay. HEK293 cells were irradiated with UV (20 J/m²) using CL-1000 UV cross-linker (UVP) and, after 48 or 72 h, fixed in 3.7% paraformaldehyde, permeabilized in 0.2% Triton X-100–phosphate-buffered saline (PBS), and subjected to terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) by using a TUNEL kit according to the manufacturer's instructions (Promega). Nuclear staining was performed with Hoechst 33258 (0.5 μ g/ml), and cells were visualized with a Biozero fluorescence microscope (Keyence). Around 500 cells in four different fields were counted per cover glass. The cell numbers were converted into percentages of apoptotic cells calculated from total cell numbers.

Flow cytometry. HEK293 synchronized cells were trypsinized, resuspended in PBS, and fixed in 70% ethanol by the addition of 100% ethanol drop by drop while shaking. Fixed cells were stained with a solution of 1 mM sodium citrate, 500 mg/ml propidium iodide, 100 mg/ml RNase A in

PBS overnight at 4°C. Cells were analyzed by using the Guava EasyCyte mini-flow cytometer and Guava Cellcycle software (Millipore) to assess cell cycle phase distribution.

GST pulldown assay. Glutathione S-transferase (GST) fusion proteins were expressed in *E. coli* strain BL-21 by using the pGEX vector system. Various GST-fused proteins immobilized on glutathione-Sepharose were incubated with cell extracts from transfected HEK293T cells or *in vitro*-translated protein (TnT system; Promega), which was diluted with binding buffer (50 mM HEPES [pH 7.9], 150 mM NaCl, 0.1% Triton X-100–protease inhibitors). After incubation for 4 h at 4°C, the beads were washed three times with the same buffer, and proteins were analyzed by Western blotting.

Single-stranded DNA pulldown assay. The ssDNA pulldown assay was performed as described previously (19) with some modifications. Biotinylated 50-mer oligonucleotide (TTGTAAAACGCGGCCAGTGAA TTCATCATCAATATTCCTTTTTTGGCAGGCGGTGTTAATACTGCC GCC) was bound to streptavidin-coupled beads (Dynal) according to the manufacturer's directions and then incubated with the purified FLAG-RPA proteins in 500 μ l of binding buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Triton X-100) at 4°C for 30 min. The beads were retrieved, washed three times with binding buffer, and incubated with cell lysate expressing hemagglutinin (HA)-FOXO1 or HA-FOXO3a proteins together with poly(dI-dC) (20 μ g/ml) at 4°C for 1 h. The beads were retrieved and washed with binding buffer, followed by Western blotting.

***C. elegans* strains.** All strains were cultured according to standard methods. The strains used were strain Bristol N2 wild type (WT), strain CF1038 *daf-16(mu86)I*, strain XF656 *polh-1(ok3317)III*, strain RB864 *xpa-1(ok698)I*, strain TJ356 *zIs356[daf-16::GFP; rol-6]*, strain TKB222 *trcIs22[daf-16WT::GFP]*; *daf-16(mu86)I*, strain TKB230 *trcIs18[daf-16GP::GFP]*; *daf-16(mu86)I*, and strain TKB231 *trcIs24[daf-16 Δ AD::GFP]*; *daf-16(mu86)I*.

Larval development assay. Synchronized first-stage larvae (L1) (about 50 worms) were exposed to UV (20 J/m²) on nematode growth medium (NGM) plates without food (*E. coli* OP50) and then transferred to NGM plates with OP50. The larvae at each stage were counted at postirradiation periods when over 80% of the nonirradiated L1 larvae of each strain developed to the fourth stage (L4).

Adult life span assay. Life span assays were conducted at 20°C. Synchronized L1 larvae (about 100 worms) were grown to young adult and then transferred to plates without OP50. Following UV irradiation at 20 J/m², worms were transferred back to OP50-seeded NGM plates containing floxuridine (0.5 mg/ml) to prevent the production of progeny. Animals were tapped every day and scored as dead when they did not respond to the platinum wire pick. All of the life span assays were repeated at least two times.

Cellular localization analysis of DAF-16–GFP in L1 larvae. Synchronized L1 larvae of TJ356 were grown on NGM plates with OP50 for 4 h at 20°C and then washed off the plates with M9 buffer and transferred to NGM plates without OP50. Following UV irradiation at 20 J/m², the worms were grown for 3 h on NGM plates with OP50 and then observed under the fluorescence microscope.

Statistical analysis. The results are presented as the mean values $\pm$ standard errors of the means (SEM). Statistically significant differences, set at a *P* value of <0.01, were determined by using the two-tailed unpaired Student *t* test.

RESULTS

FOXO1 knockdown cells are highly sensitive to UV. To examine the role of FOXO transcription factors in response to UV-induced DNA damage, we first measured apoptotic cell death with the TUNEL method. Two days after siRNA transfection, HEK293 cells were left unexposed or exposed to UV irradiation at a dose of 20 J/m² and further incubated for 72 h. As shown by the results in Fig. 1A, control siRNA-transfected cells showed only slight apoptosis (less than 5%) after exposure to UV. In contrast, FOXO1

FIG 1 Knockdown of FOXO1 but not FOXO3a causes high UV sensitivity. (A) Silencing FOXO1 results in UV-induced apoptosis. HEK293 cells transfected with control or FOXO1 siRNAs were exposed to UV (20 J/m²), and then apoptotic cells were determined with the TUNEL assay. The number of TUNEL-positive cells per field was calculated as the mean of the values from 10 fields. Error bars represent the standard errors. (B) UV-induced apoptosis was confirmed by detecting cleaved PARP1. Whole-cell lysates from HEK293 cells treated as described in the legend to panel A were immunoblotted with antibodies to the indicated proteins. (C) Silencing FOXO3a has no effect on UV-induced apoptosis. (Top) RNA interference (RNAi) specificity was confirmed by Western blotting. (Bottom) UV-induced apoptosis in HEK293 cells transfected with indicated siRNAs was determined with the TUNEL assay as described in the legend to panel A. (D) Silencing GADD45 has no effect on UV-induced apoptosis. Data are presented as the mean values $\pm$ standard errors of the means (SEM). *, $P < 0.01$; significantly different from the value obtained in control knockdown cells exposed to UV.

knockdown with two distinct siRNA duplexes enhanced UV-induced apoptosis by 35 and 40%, respectively (Fig. 1A). Similar results were obtained in human breast cancer MCF-7 cells (data not shown). The UV-induced apoptosis by FOXO1 depletion was further confirmed by detection of the cleaved form of poly(ADP-ribose) polymerase 1 (PARP1), a typical caspase-3 substrate (Fig. 1B). Next, we investigated whether another FOXO subfamily member, FOXO3a, is redundantly responsible for UV resistance. Interestingly, however, FOXO3a knockdown had no effect on cell survival after exposure to UV, indicating that there is a functional difference between FOXO1 and FOXO3a in UV resistance (Fig. 1C).

Moreover, because the FOXO factors have been shown to facilitate the DNA damage response by upregulating gene expression for GADD45, a nuclear protein involved in genomic stability,

DNA repair, and suppression of cell growth (20), we tested whether GADD45 could be implicated in UV-induced apoptosis. A substantial reduction of GADD45 protein was accomplished by using GADD45-specific siRNA, and a further partial reduction was achieved with FOXO1-specific siRNA, likely due to a decline in FOXO1-induced GADD45 transcription (Fig. 1D, top). However, the UV sensitivity of GADD45 knockdown cells was equivalent to that of control cells (Fig. 1D, bottom), suggesting that the involvement of FOXO1 in UV resistance is not due to upregulation of GADD45 expression. Alternatively, one mechanism by which cells protect themselves against UV irradiation is the removal of UV-induced DNA lesions, such as CPD and 6-4PP, by nucleotide excision repair (NER) (10). Thus, we examined the possibility that the UV sensitivity caused by FOXO1 depletion is indicative of impaired NER. Both control and FOXO1 knockdown cells showed a slight decrease in the amount of CPD even at 24 h post-UV irradiation, but there were no significant differences (see Fig. S1 in the supplemental material, top). Unlike CPD, 6-4PP was readily removed at 2 h post-UV irradiation, but the efficiency of 6-4PP removal was almost the same in control and FOXO1 knockdown cells (see Fig. S1, bottom). These results indicate that FOXO1 does not participate in the excision of UV-damaged DNA. Taking the results together, we next attempted to determine the DNA repair machinery that FOXO1 is involved in.

FOXO1 knockdown abrogates the progression of S phase and cell proliferation after UV irradiation. The choice of which repair machinery to use is dependent on both the type of DNA lesion and the cell cycle stage where the response is elicited (21). We therefore synchronized HEK293 cells transfected with control or FOXO1 siRNA at four different stages, G₁ phase, S phase, the G₂/M boundary, and M phase, and then exposed the cells at each stage to UV at 20 J/m². TUNEL assay revealed that UV irradiation at G₁ and M phases had no effect on cell viability in either control or FOXO1 knockdown cells (Fig. 2A). In contrast, a substantial level of UV-induced apoptosis was observed in S phase and a lower level in G₂/M phase when FOXO1 was silenced. These results raised the possibility that FOXO1 is implicated in the intra-S DNA damage checkpoint in response to UV irradiation.

Once UV-induced CPDs are left unrepaired during S phase, they usually block the progress of replication forks catalyzed by DNA polymerases. However, in general, cells have developed a damage tolerance mechanism called translesion DNA synthesis (TLS), in which DNA polymerase η (Pol η), a member of the mammalian Y family polymerases, can replicate past CPDs by incorporating correct bases on the opposite strand and thereby overcome replication blocks (11). Hence, if FOXO1 plays a role in the TLS process, the S-phase progression of FOXO1 knockdown cells could be delayed or prevented following UV exposure. As expected, we found that FOXO1 knockdown cells exhibited a UV-induced delay in S-phase progression compared with the progression of the control knockdown cells (Fig. 2B), while no difference was observed in the absence of UV exposure (see Fig. S2 in the supplemental material). To further investigate whether FOXO1 is indeed involved in TLS, the UV sensitivities of FOXO1 and/or Pol η knockdown cells were determined with a colony formation assay after exposure to 4 and 8 J/m² UV. Compared with control cells, FOXO1 knockdown cells were highly sensitive to UV irradiation, showing a rate of survival similar to that of Pol η knockdown cells (Fig. 2C). It is noted that a double knockdown of FOXO1 and Pol η did not decrease the surviving fraction of cells

FIG 2 Knockdown of FOXO1 blocks the progression of S phase and cell proliferation after UV irradiation. (A) UV irradiation at S phase causes apoptosis in FOXO1 knockdown cells. HEK293 cells transfected with control or FOXO1 siRNA were synchronized at G₁ phase, S phase, the G₂/M boundary, and M phase, followed by exposure to UV (20 J/m²), and then apoptotic cells were determined with the TUNEL assay. The number of TUNEL-positive cells per field was calculated as the mean of the values from 10 fields. Data are presented as mean values $\pm$ standard errors of the means (SEM). *, $P < 0.01$; significantly different from the value obtained in control knockdown cells exposed to UV. (B) Silencing FOXO1 results in UV-induced delay in S-phase progression. HEK293 cells transfected with the indicated siRNAs were synchronized at S phase. After exposure to UV (20 J/m²), the progression of S phase was determined by flow cytometry. (C) Silencing FOXO1 as well as Pol η decreases cell proliferation after UV irradiation. UV sensitivities of HeLa cells transfected with the indicated siRNAs were determined with a colony formation assay after exposure to UV at 4 and 8 J/m². Data are presented as mean values $\pm$ standard errors of the means (SEM).

exposed to UV irradiation compared with single-gene knockdown. Taken together, our findings suggest that FOXO1 and Pol η are functionally related.

FOXO1 knockdown promotes sustained activation of ATR-Chk1 signaling after UV irradiation. Because defects in TLS result in stalled replication forks at CPD sites and then ATR phosphorylates/activates the effector kinase Chk1, we tested the possible involvement of FOXO1 in TLS by measuring ATR-induced phosphorylation of Chk1 at serine 345. HEK293 cells transfected with control or FOXO1 siRNA were synchronized in early S phase, exposed to UV, and then further incubated for 2, 6, or 9 h. As shown by the results in Fig. 3A, knockdown of FOXO1 led to a marked increase in the phosphorylation levels of Chk1 at 2 h post-UV exposure and also caused a subsequent sustained Chk1

phosphorylation until 9 h post-UV exposure, when the phospho-Chk1 signals had completely disappeared in control cells. It should be noted that, consistent with a previous report (22), Pol η knockdown cells showed a similar increased and sustained phosphorylation of Chk1 following UV irradiation (Fig. 3B). In contrast, Chk1 hyperphosphorylation by knockdown of FOXO1 or Pol η was not observed either when cells were irradiated with UV in G₁ phase or when cell proliferation was blocked by treatment with hydroxyurea (HU) (Fig. 3C and D). Collectively, these findings suggest that FOXO1 depletion results in sustained activation of ATR signaling in response to UV-induced replication block, probably due to defects in TLS.

Unlike knockdown of FOXO1, knockdown of FOXO3a failed to alter the phosphorylation levels of Chk1 (Fig. 3E). On the other

FIG 3 Knockdown of FOXO1 promotes sustained phosphorylation of Chk1 after UV irradiation. (A and B) Silencing FOXO1 or Pol η results in enhanced and sustained phosphorylation of Chk1 after UV irradiation during S phase. HEK293 cells transfected with the indicated siRNAs were synchronized at S phase. After exposure to UV (20 J/m²), whole-cell lysates were immunoblotted with antibodies to the indicated proteins. (C) Silencing FOXO1 or Pol η has no effect on phosphorylation of Chk1 after UV irradiation during G₁ phase. HEK293 cells transfected with the indicated siRNAs were synchronized at G₁ phase and treated with or without UV (20 J/m²), and after 6 h, whole-cell lysates were immunoblotted with antibodies to the indicated proteins. (D) Silencing FOXO1 has no effect on phosphorylation of Chk1 after treatment with hydroxyurea (HU; 1 mM) during S phase. HEK293 cells transfected with the indicated siRNAs were synchronized at S phase. After treatment with HU, whole-cell lysates were immunoblotted with antibodies to the indicated proteins. (E) Silencing FOXO3a has no effect on phosphorylation of Chk1 after UV irradiation during S phase. (F) Silencing FOXO3a but not FOXO1 leads to a decrease in phosphorylation of histone H2AX and ATM after UV irradiation during S phase. HEK293 cells transfected with the indicated siRNAs were synchronized at S phase and treated with or without UV (20 J/m²), and after 6 h, chromatin fractions and whole-cell lysates were immunoblotted with antibodies to the indicated proteins.

hand, FOXO3a has been reported to promote autophosphorylation/activation of ataxia telangiectasia mutated (ATM) by direct interaction and, thus, activate its downstream mediators to control damage-induced cell cycle checkpoints and DNA repair (23). To evaluate the role of FOXO1 in the ATM-mediated DNA damage response, HEK293 cells transfected with control, FOXO1, or FOXO3a siRNA were exposed to UV irradiation and their lysates were analyzed by phosphor-ATM (Ser1981) and phosphor-histone H2AX (γ -H2AX) antibodies. In agreement with a previous study using ionizing radiation (23), knockdown of FOXO3a abrogated phosphorylation of both ATM and histone H2AX, perhaps due to the formation of replication-associated double-strand breaks following UV damage, while knockdown of FOXO1 had no effect on their phosphorylation status compared with the results for the control knockdown (Fig. 3F). These results indicate that, in

contrast to FOXO3a, FOXO1 could regulate ATR signaling downstream from the UV damage response.

FOXO1 is partially required for PCNA monoubiquitination but not for Pol η focus formation. Since PCNA monoubiquitination triggers a switch from the replicative polymerase blocked at a lesion to Pol η , we first studied the effects of FOXO1 depletion on PCNA monoubiquitination. HEK293 cells transfected with control or FOXO1 siRNA were synchronized in early S phase and then exposed to 20 J/m² UV, and 2, 4, or 6 h later, the chromatin fraction and whole-cell lysates were analyzed by Western blotting. As shown by the results in Fig. 4A, knockdown of FOXO1 resulted in a slight but significant decrease in the monoubiquitination level of PCNA at all periods after UV irradiation. Furthermore, the amount of RAD18 protein in the chromatin fraction was also diminished by FOXO1 depletion, suggesting that UV-induced

FIG 4 FOXO1 is required for PCNA monoubiquitination but not for Pol η focus formation. (A) FOXO1 is partially responsible for UV-inducible PCNA monoubiquitination. HEK293 cells were transfected with the indicated siRNAs and treated with or without UV (20 J/m²), and after 2, 4, and 6 h, chromatin fractions and whole-cell lysates were immunoblotted with antibodies to the indicated proteins. (B) FOXO1 is not required for UV-inducible focus formation of Pol η . HEK293 cells were sequentially transfected with the indicated siRNAs and GFP-Pol η , treated with or without UV (20 J/m²), and after 6 h, fixed and observed by fluorescence microscopy. DAPI, 4',6-diamidino-2-phenylindole. (C) Quantification of cells displaying more than 20 GFP-Pol η foci in the experiment whose results are shown in panel B. Data are presented as mean values $\pm$ standard errors of the means (SEM). *, $P < 0.01$; significantly different from the value obtained in control knockdown cells exposed to UV.

RAD18 loading is influenced by FOXO1 (see Fig. S3 in the supplemental material).

In order to further examine the involvement of FOXO1 in TLS, we next focused on the subcellular translocation of Pol η after exposure to UV (13). We observed that transiently expressed GFP-Pol η was localized uniformly in the nucleus of control knockdown cells at basal state and actually accumulated into nuclear foci following UV irradiation (Fig. 4B and C). The formation of foci was markedly abolished by knockdown of RAD18, whereas FOXO1 depletion had no effect on the UV-induced assembly of Pol η foci (Fig. 4B and C). Taken together, these results indicate that FOXO1 is partially required at least for PCNA monoubiquitination but not for the formation of Pol η foci in the initiation of TLS.

FOXO1 does not regulate the expression of TLS-related genes but binds to RPA1. To clarify the molecular mechanism underlying the contribution of FOXO1 to the TLS pathway, we first tested the possibility that FOXO1 regulates the gene expression of Pol η and its related proteins, such as the clamp protein PCNA, the clamp loader complex CTF18-RFC, and the ssDNA-binding protein RPA, all of which have been shown to stimulate the DNA-synthetic activity of Pol η (15, 16). However, neither knockdown of FOXO1 nor transfection of the constitutively active mutant FOXO1(3A), in which all three Akt phosphorylation sites are replaced with alanine, altered the expression levels of Pol η ,

CTF18, PCNA, RPA1, RPA2, and RAD18 in HEK293 cells (Fig. 5A; see also Fig. S4 in the supplemental material). These results indicate that the contribution of FOXO1 to TLS is likely not dependent on the expression of TLS-related genes.

Instead, given the physical interaction of FOXO3a with ATM, as mentioned above (23), we investigated whether FOXO1 could form a complex with the TLS machinery, including Pol η , PCNA, RPA1, and CTF18. A coimmunoprecipitation assay showed that endogenous FOXO1 binds specifically to RPA1 in HEK293 cells (Fig. 5B). Because RPA1 is a subunit of the RPA heterotrimeric complex, together with RPA2 and RPA3 (24), we attempted to determine which components bind to FOXO1 by preparing bacterially expressed GST-FOXO1 and whole-cell lysates from cells expressing fusions of the yellow fluorescent protein Venus with RPA1, RPA2, and RPA3. GST pull-down assays demonstrated that FOXO1 preferentially binds to RPA1 *in vitro* (Fig. 5C). Furthermore, we constructed a series of deletion mutants of FOXO1 and found that the forkhead DNA-binding domain is responsible for interacting with RPA1 (Fig. 5D). To confirm this result, we generated the FOXO1(GP) mutant, harboring two point substitutions (W206G and H212P) that impair the structure of the forkhead domain and thereby result in a significant reduction of the DNA-binding activity (25). As shown in Fig. 5E, as well as Fig. S4B in the supplemental material, the GP mutation completely abol-

FIG 5 FOXO1 is not involved in TLS-related gene expression but binds to ssDNA by interacting with RPA1. (A) FOXO1 does not regulate the expression of TLS-related genes. Whole-cell lysates from HEK293 cells transfected with control or FOXO1 siRNA were immunoblotted with antibodies to the indicated proteins. (B) Endogenous FOXO1 interacts with RPA1. Whole-cell lysates from HEK293 cells were immunoprecipitated with normal IgG or anti-FOXO1 antibody, followed by Western blotting with antibodies to the indicated proteins. (C) FOXO1 specifically interacts with RPA1 *in vitro*. Whole-cell lysates from HEK293T cells expressing Venus-RPA1, -RPA2 or -RPA3 were incubated with GST or GST-FOXO1, followed by Western blotting (WB) with anti-Venus antibody. GST fusion proteins are shown by Coomassie brilliant blue (CBB) staining. (D) Forkhead domain of FOXO1 is required for RPA1 binding. (Left) Schematic of FOXO1 deletions. FHD, forkhead domain; AD, activation domain; NT, N terminus; FH, forkhead; M, middle region; CT, C terminus. (Right) Whole-cell lysates from HEK293T cells expressing HA-RPA1 were incubated with GST or GST-FOXO1, followed by Western blotting with anti-HA antibody. GST proteins are shown by CBB staining. (E) FOXO1(GP) mutation abolishes the interaction with RPA1. Whole-cell lysates from HEK293T cells expressing HA-RPA1 were incubated with GST, GST-FOXO1 wild-type, or the GP mutant, followed by Western blotting with anti-HA antibody. GST proteins are shown by CBB staining. (F) FOXO1 is recruited to ssDNA in a manner dependent on RPA1. ssDNA-conjugated beads were preincubated with increasing amounts of FLAG-RPA1 and then incubated with whole-cell lysates from HEK293T cells expressing HA-FOXO1 or -FOXO3a, followed by Western blotting with antibodies to the indicated proteins.

ished the FOXO1-RPA1 interaction *in vitro* and *in vivo*. Furthermore, we identified the C terminus of RPA1, called DBD-C, as a binding region for FOXO1 (see Fig. 4C). RPA is known to not only bind and stabilize ssDNA regions during DNA replication and repair but also recruit a variety of DNA-processing proteins through direct interaction (24). Therefore, we tested whether FOXO1 could be recruited to an ssDNA region through interaction with RPA1. We found that FOXO1 fails to directly bind to ssDNA, whereas the addition of RPA1 enables FOXO1 to bind to ssDNA in a dose-dependent manner, indicating that the recruitment of FOXO1 to ssDNA is entirely dependent on the presence of

RPA1 (Fig. 5F). In addition, although FOXO3a also bound to RPA1-ssDNA, this interaction was at a relatively low level in comparison with that of FOXO1 (Fig. 5F). Taken together, these results suggest that RPA1 could be a candidate scaffold protein for FOXO1 to participate in the TLS process.

FOXO/DAF-16 is required for UV tolerance during larval development in *C. elegans*. The possible involvement of FOXO1 in TLS led us next to determine whether this function could be conserved across species. A genetic study with the nematode *Caenorhabditis elegans* is often used for evaluating the functions of the FOXO ortholog DAF-16 and has actually provided evidence that

several longevity pathways converge on DAF-16 (26). In this study, since TLS is accompanied by DNA replication, we focused on *C. elegans* larvae, whose somatic tissues are composed of proliferative cells, and sought to assess the effect of UV-induced DNA damage on the progression of larval development from L1 to L4 (Fig. 6A). First, to address whether DAF-16 responds to UV irradiation during L1, we observed the cellular localization of DAF-16–GFP *in vivo* (27). Although DAF-16–GFP was normally localized in the cytoplasm without UV, we found a predominantly nuclear localization after exposure to 20 J/m² UV in L1 larvae (Fig. 6B). Next, the wild-type strain N2, a *daf-16*-null mutant [*daf-16(mu86)*], and a mutant that was null for the *C. elegans* ortholog of *polh* [*polh-1(ok3317)*] were synchronized to L1 larvae and immediately exposed to 20 J/m² UV. Thereafter, when over 80% of the animals in each control treatment (no UV) had become L4 larvae, the developmental stages of the corresponding UV-irradiated animals were categorized as L1/L2, L3, and L4. We found that UV irradiation caused a substantial and an almost complete arrest of larval development in the *daf-16(mu86)* and *polh-1(ok3317)* mutants, respectively, while only a slight retardation was observed in N2 animals (Fig. 6C). These results suggest that DAF-16 contributes to tolerance of UV-induced DNA damage in larval development, albeit with a lower contribution than POLH-1.

Since, unlike the larval stages, the somatic tissues in the adult animals are quiescent and composed of postmitotic cells, the UV sensitivity of adult *C. elegans* worms was predicted not to be dependent on the TLS process. To test this hypothesis, we evaluated the UV resistance of N2, *daf-16(mu86)*, and *polh-1(ok3317)* adults, together with a mutant with a loss-of-function mutation in the NER pathway [*xpa-1(ok698)*], using 1-day-old adults with or without UV irradiation. As expected, we found that N2 animals and *daf-16(mu86)* and *polh-1(ok3317)* mutants exhibited no significant differences in the survival curves of UV-irradiated and unirradiated animals (Fig. 6D to F). Importantly, consistent with a previous report (28), the *xpa-1(ok698)* mutants were highly sensitive to UV during adulthood, likely due to the lack of NER (Fig. 6G). Collectively, these results suggest that DAF-16, as well as POLH-1, plays a critical role in TLS but not in NER in *C. elegans*.

FOXO/DAF-16 contributes to UV tolerance during larval development independently of its transactivation function. Finally, to explore which functions of DAF-16 are required for UV tolerance during larval development, we attempted to perform rescue experiments with transgenic lines expressing wild-type DAF-16 or one of two DAF-16 mutants, in the *daf-16(mu86)* background. The DAF-16GP mutant harbors point mutations at the conserved tryptophan (W) and histidine (H) residues within the forkhead DNA-binding domain [corresponding to FOXO1(GP)], while the DAF-16ΔAD mutant lacks the C terminus (residues 473 to 510), which is primarily responsible for the transactivation function (Fig. 7A) (29). We first confirmed that the DAF-16GP mutant, but not the DAF-16ΔAD mutant, was unable to interact with RPA1, the *C. elegans* ortholog of RPA1, in a GST pulldown assay (Fig. 7B). In addition, we conducted a luciferase reporter assay in HEK293T cells using the wild-type protein and the two DAF-16 mutants and demonstrated that not only the ΔAD mutant but also the GP mutant that is impaired in the DNA-binding domain does not induce the transcription of a reporter gene (Fig. 7C). Thereafter, we generated three integrated lines, with genotypes *trcIs35[daf-16wt(+):gfp]*; *daf-16(mu86)*, *trcIs36[daf-16ΔAD(+):gfp]*; *daf-16(mu86)*, and *trcIs18[daf-*

16GP(+):gfp]; *daf-16(mu86)*, and examined the effect of UV-induced DNA damage on the progression of larval development. As shown by the results in Fig. 7D, transgenic rescue with the *daf-16WT* gene entirely restored the progression of larval development of *daf-16(mu86)* mutants after UV irradiation. Most importantly, a similar improvement was also observed in *Is36[daf-16ΔAD(+):gfp]*; *daf-16(mu86)* animals, whereas the *daf-16GP* gene failed to rescue the decreased UV tolerance in the *daf-16(mu86)* background. Taken together, these data suggest that DAF-16-dependent UV tolerance in larval development requires not the transactivation function but the RPA1-binding activity of DAF-16.

DISCUSSION

In the current study, we demonstrate that FOXO1 has a role in translesion DNA synthesis, in addition to its known role as a transcription factor. FOXO1 contributes to the UV damage response when cells are irradiated specifically during S phase and is partially required for UV-inducible RAD18 loading and PCNA monoubiquitination but not for Polη focus formation. Accordingly, knockdown of FOXO1 results in the sustained activation of ATR–Chk1 signaling after UV irradiation, probably due to defects in TLS. Moreover, FOXO1 is able to bind to a single-stranded DNA by interacting with RPA1 through the forkhead domain. In *C. elegans*, DAF-16/FOXO plays a key role in the tolerance of UV-induced DNA damage during larval development. Importantly, rescue experiments with functional mutants of DAF-16 revealed that the forkhead domain that is responsible for RPA1 binding, but not the transactivation domain, could be necessary for the UV tolerance ability of DAF-16/FOXO.

Our present results imply that FOXO1/DAF-16 contributes to TLS following UV irradiation independently of its transactivation function. How does FOXO1/DAF-16 influence UV tolerance during DNA replication? Interestingly, several reports have established chromatin remodeling as a new regulatory process in relatively early steps of TLS. For example, in yeast, two distinct chromatin-remodeling complexes, INO80 and RSC, promote RAD18 recruitment at stalled replication forks, thereby facilitating PCNA ubiquitination and TLS (30, 31). Additionally, in mammals, the transcriptional repressor ZBTB1 has been shown to control KAP-1-dependent chromatin remodeling to promote UV-inducible PCNA monoubiquitination during TLS (32). Given our finding that FOXO1 knockdown reduced PCNA monoubiquitination (Fig. 4A; see also Fig. S3 in the supplemental material), FOXO1/DAF-16 could be involved in recruiting a chromatin-remodeling complex to stalled replication forks through interaction with ssDNA-bound RPA1. Supporting this idea, a recent study has reported that DAF-16 employs the chromatin remodeling proteins SWI/SNF as cofactors to regulate its target genes and increases stress resistance and longevity (33). Instead, it should be noted that FOXO1 itself is known to be able to disrupt core histone–DNA contacts and open compacted chromatin arrays in the IGFBP-1 promoter (34), raising the possibility that the chromatin-opening ability of FOXO1 is solely sufficient to facilitate TLS. Further studies are needed to elucidate the relationship between FOXO1/DAF-16-mediated UV tolerance and chromatin remodeling.

Our findings from biochemical and transgenic rescue experiments lead us to argue that FOXO1/DAF-16 contributes to DNA damage tolerance by forming a complex with RPA1 through the

FIG 6 DAF-16 contributes to UV tolerance during larval development but not the adult life span in *C. elegans*. (A) Schematic of the life cycle of *C. elegans*. *C. elegans* passes through four larval stages (L1 to L4) to reach adulthood. (B) GFP-DAF-16 accumulates in the nucleus after exposure to UV during L1. Synchronized L1 larvae of strain TJ356 were exposed to UV (20 J/m²) and, after 3 h, observed by fluorescence microscopy. (C) Effects of UV irradiation on larval development of strain N2 and *daf-16* and *polh-1* mutants. Synchronized L1 larvae were exposed to UV (20 J/m²), and when over 80% of animals in each control treatment (no UV irradiation) had developed into L4 larvae, the UV-treated animals in L1/L2, L3, and L4 developmental stages were counted. Data are presented as mean values $\pm$ standard errors of the means (SEM). *, $P < 0.01$; significantly different from the value obtained for the L1/L2 fraction of strain N2 exposed to UV. (D to G) Effect of UV irradiation on adult life span. Survival of strain N2 and *daf-16*, *polh-1*, and *xpa-1* mutants with or without UV irradiation (50 J/m²) was measured.

FIG 7 Transactivation function of DAF-16 is not required for UV tolerance during larval development. (A) Schematic of DAF-16 mutants. FHD, forkhead domain; AD, activation domain; GP, W193G H199P double mutation. (B) DAF-16GP but not Δ AD mutant fails to bind to *C. elegans* RPA1. *In vitro*-translated HA-RPA1 was incubated with GST or GST-DAF-16WT, -GP, or Δ AD, followed by Western blotting with anti-HA antibody. GST proteins are shown by CBB staining. (C) Both the DAF-16GP and Δ AD mutant fail to activate the reporter gene. HEK293T cells were transfected with a luciferase reporter construct (3xIRS-MLP-luc), together with increasing amounts of DAF-16WT, -GP, or Δ AD. After 48 h, luciferase activities were measured, and the results are presented as arbitrary units. (D) Effects of UV irradiation on larval development of transgenic animals. Synchronized L1 larvae were exposed to UV (20 J/m²), and when over 80% of the animals in each control treatment (no UV irradiation) had developed into L4 larvae, the UV-irradiated animals in L1/L2, L3, and L4 developmental stages were counted. Data are presented as mean values $\pm$ standard errors of the means (SEM). *, $P < 0.01$; significantly different from the value obtained in cells transfected with empty vector (C) or in the L1/L2 fraction of *Is[daf-16WT(+)] ; daf-16(mu86)* animals exposed to UV (D). n.s., not statistically significant.

forkhead domain and, also, propose a model in which the transcription factor FOXO1/DAF-16 participates in an inherent function of RPA, namely, the binding and stabilizing of ssDNA regions during TLS. In contrast, a recent study has demonstrated that RPA1 helps in the recruitment of transcription factor HSF1 to nucleosomal DNA by recruiting the histone chaperone FACT and, thus, enables constitutive HSF1 access to nucleosomal DNA for both basal and inducible gene expression (35). Taken together, these results provide the possibility that RPA1 and transcription factors mutually affect and regulate each other's functions, including DNA repair, DNA replication, and transcription. Indeed, since RPA1 strongly represses the transcriptional activity of FOXO1 (data not shown), presumably through masking the forkhead DNA-binding domain, RPA1 interaction may be a trigger for the switching of FOXO1 function from transcriptional activation to DNA damage tolerance.

FOXO1 has been shown to undergo posttranslational modifications, including phosphorylation, acetylation, ubiquitination, lysine and arginine methylation, and glycosylation, which can regulate the transactivation function of FOXO1 by modulating its

subcellular localization, protein stability, DNA binding, transcriptional activity, and interaction with other proteins (2, 3, 36). In addition, we previously reported that poly(ADP-ribose)polymerase 1 (PARP1) binds and poly(ADP-ribosyl)ates FOXO1; however, the functional significance of poly(ADP-ribosyl)ation remains unclear (37). It is well established that, following DNA damage, PARP1 senses and binds to DNA single and double-strand breaks, whereby it becomes activated and catalyzes the attachment of poly(ADP)ribose polymers onto substrates, including transcription factors, histones, and PARP1 itself (38). Besides opening chromatin, poly(ADP)ribosylation causes PARP1 dissociation from DNA, allowing access to an orchestrated network of repair enzymes involved in base excision repair, nucleotide excision repair, mismatch repair, and DNA double-strand-break repair. In particular, a recent study reported that PARP10, a mono(ADP-ribosyl) transferase, interacts with PCNA and is required for DNA damage tolerance (39). Although the relationship between FOXO1 and PARP10 in TLS is unknown, these findings, together with our results presented here, suggest a possible involvement of PARP activity in FOXO1/DAF-16-mediated UV tolerance.

Here, we provide evidence that impaired function of FOXO1 results in sustained activation of the ATR-Chk1 axis after UV irradiation during S phase (Fig. 3A). On the other hand, consistent with a previous report (23), we found that FOXO3a promotes autophosphorylation of ATM and, thus, phosphorylates downstream H2AX even when irradiated with UV (Fig. 3F). These results demonstrate that although FOXO1 and FOXO3a appear to have both distinct and overlapping functions that could compensate each other, there is a functional difference in their transactivation-independent DNA damage responses. In addition, considering that ATR disruption leads to early embryonic lethality, unlike the ATM disruption in ATM-null mice (40, 41), our findings may provide an explanation for the distinct phenotypes of two FOXO knockout mice; FoxO1-null mice die at embryonic day 10.5, while FoxO3-null mice are viable and have no apparent phenotype (42).

In *C. elegans*, *daf-16*, as well as *polh-1*, is required for UV tolerance during larval development but not during adulthood (Fig. 6). These different resistance patterns among stages could be attributed to whether the somatic cells proliferate when irradiated with UV. Since life span is typically defined as the number of days after the adult molt and during which *C. elegans* consists only of postmitotic cells, with the exception of germ line precursor cells, it seems likely that DAF-16-mediated longevity occurs independently of its TLS function. In support of this idea, *Polh*-deficient mice are viable and do not show any obvious spontaneous defects, such as a premature aging-like phenotype, at least during the first year of life (43). Alternatively, given that these mutant mice are highly susceptible to developing skin carcinomas following chronic exposure to UV irradiation, FOXO1-mediated UV tolerance may also play a role in decreasing the risk of genomic instability by preventing stalled replication forks from degenerating into defective DNA structures.

Recently, Mueller et al. reported that DAF-16 alleviates DNA damage-induced developmental arrest and promotes developmental growth even in the absence of nucleotide excision repair (44). They argue that DAF-16 is activated in response to persistent DNA damage and induces somatic growth genes through binding to the GATA transcription factor EGL-27, whose zinc finger domain in turn recognizes the consensus sequence overlapping GATA and the DAF-16-associated element (DAE) in the target gene promoter. Most importantly, however, developmental growth, especially when overriding persistent DNA lesions, requires TLS, and our present findings provide a mechanism whereby DAF-16 binds to RPA1 and facilitates TLS without transcriptional activation. Thus, these dual functions of DAF-16 would cooperatively play a critical role in UV tolerance during larval development.

In conclusion, here, we present evidence that FOXO1/DAF-16 contributes to tolerance of UV-induced DNA damage independently of its transcriptional activity in mammalian cells and *C. elegans*. This conserved mechanism was not relevant to longevity in *C. elegans* but, alternatively, may be involved in other FOXO functions, particularly functions such as stem cell maintenance in mammals (45, 46). If so, in view of the notion that aberrant stem cell function is a hallmark of aging (47), our findings will provide new insights into the molecular mechanisms of aging and the pathogenesis of age-related diseases, including type 2 diabetes, Alzheimer's disease, and cancer.

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